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Characterization of the DNA of a Defective Human Parvovirus Isolated from a Genital Site

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An apparently helper-dependent parvovirus was isolated from a penile flat condylomatous lesion by inoculating the material into a culture of human fibroblasts, and by coinfecting these cells with adenovirus type 12. Upon addition of an adenovirus helper, the virus could be readily propagated in human KB cells. Analysis of its DNA revealed that it shares structural similarities with the DNA of the four defective parvoviruses (adeno-associated viruses, AAV) described so far, but that the cleavage pattern obtained after digesting the DNA with restriction enzymes is different from that of AAV 1–AAV 4. Nucleic acid hybridization data further support the assumption that the isolated defective parvovirus is not identical with the other four serotypes. When conditions of high stringency were used, only weak cross-reactivity was seen with the DNAs of AAV 1–AAV 4, whereas the latter showed strong cross-hybridization with each other. The organization of the DNA of the newly isolated parvovirus was investigated by mapping the cleavage sites of several restriction enzymes. It is proposed to designate the new isolate as AAV 5.

INTRODUCTION

In the course of experiments to study the possible influence of adenovirus infection on replication of papilloma viruses in tissue culture cells, an apparently helper-dependent small virus was isolated from a penile flat condylomatous lesion. From its size and the observed helper dependence on adenoviruses for its replication, it was concluded to be an adeno-associated virus (AAV).

Adeno-associated viruses are members of the parvoviridae family and are defective in that they require coinfection with adenovirus or herpesvirus for productive infection (Atchison *et al.*, 1965; Hoggan *et al.*, 1966; Buller *et al.*, 1981). The genome of AAV is a linear, single-stranded DNA with the complementary strands separately encapsidated into individual virions with equal frequency. Upon proper conditions, these strands anneal to form linear duplex molecules (Mayor *et al.*, 1969; Rose *et al.*, 1969; Berns and Rose, 1970; Berns and Adler, 1972).

Adeno-associated viruses are interesting in several respects. Since the DNA is small (about 4.7 to 4.8 kb in size; Muster *et al.*, 1980), it provides a good model system to study genome organization and gene expression in eucaryotes. Furthermore, it shares characteristics with insertion elements in that its genome has both an inverted and a direct terminal repetition (Gerry *et al.*, 1973; Koczot *et al.*, 1973; Fife *et al.*, 1977; Lusby *et al.*, 1980; Lusby *et al.*, 1981). Actually, when no helper virus is present, AAV DNA can persist in cells of at least some continuous cell lines from which it can be rescued upon addition of helper virus (Cheung *et al.*, 1980).

In addition to these features, adeno-associated viruses have been reported to interfere with virally induced oncogenicity and cell transformation (Kirschstein *et al.*, 1968; Casto and Goodheart, 1972; Mayor *et al.*, 1973; Blacklow *et al.*, 1978; de la Maza and Carter, 1981; Ostrove *et al.*, 1981). The same holds true for the nondefective par-

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voviruses which inhibit chemically and virally induced oncogenesis and cell transformation as well as spontaneously occurring tumors (Toolan, 1967; Toolan and Ledinko, 1968; Mousset and Rommelaere, 1982; Toolan *et al.*, 1982).

In this report we describe that the small particles isolated from the genital lesion belong to the group of defective parvoviruses with regard to their helper dependence and the DNA structure. Comparison of the DNA shows that the virus differs from the four AAV serotypes (AAV 1–AAV 4) that have been found in man and monkey so far (Atchison *et al.*, 1965; Hoggan *et al.*, 1966; Parks *et al.*, 1967), not only in the size of its DNA but also in DNA homology. The new isolate is tentatively designated as AAV 5.

MATERIAL AND METHODS

Cells. Human foreskin fibroblasts and KB cells were maintained in Eagle's MEM containing 5% fetal calf serum (FCS), streptomycin, and penicillin, and supplemented in addition with glutamine. The cells were grown in monolayers and were subcultured once or twice a week. The conditions for cultivating of 293-31 cells (Graham *et al.*, 1977) and the African green monkey kidney cells (GMK) were essentially the same except for the amounts of FCS added (10% for 293-31 cells and 3% for GMK cells, respectively).

Viruses and virus infections. AAV 1 (helper: adenovirus type 7a), AAV 2 H (helper: adenovirus type 2), AAV 3 (helper: adenovirus type 2) and AAV 4 (helper: SV 15) were obtained from the American Type Culture Collection (ATCC). Cells used for propagation of virus were 293-31 for AAV 1 (helper: adenovirus type 7a); GMK for AAV 4 (helper: SV 15); and KB for AAV 2 H (helper: adenovirus type 2), AAV 3 (helper: adenovirus type 2), and AAV 5 (helper: adenovirus type 12). KB cells were also used for propagation of adenoviruses type 2 and 12. The adenovirus type 12 stock was assayed to be free of AAV 5 by using nucleic acid hybridization techniques.

The AAV inoculum was kept at 56° for 30 min to inactivate the helper virus, and new helper virus was added for coinfections. Adsorption of virus suspension was for 1 hr and then fresh medium was added. The cells were incubated at 37° for approximately 3 days. At this time, most of the cells showed cytopathogenic changes. Cells and culture fluid were subjected to three rounds of freezing and thawing and then used for virus purification.

Virus purification. Cell debris was removed by low-speed centrifugation and ammonium sulfate up to 40% saturation was added to the supernatant. The resulting precipitate was sedimented by centrifugation, redissolved in phosphate-buffered saline (PBS), and dialyzed against this buffer. The material obtained after dialysis was kept at 56° for 30 min, and was then treated with DNAse (Boehringer, Mannheim, 2000 U/mg) and RNAse (Merck, Darmstadt, 25 U/mg) at 37° for 1 hr (2 μg/ml of each enzyme). The resulting suspension was overlaid a CsCl cushion (14 ml CsCl, $\rho_{CsCl} = 1.3$ g/cm^3) and sedimented by centrifugation in a Beckman SW 27 rotor for 16 hr at 16,000 rpm. The pellet was resuspended and the suspension was brought to a density of $\rho_{CsCl} = 1.22$ g/cm^3, and then centrifuged in the Beckman SW 41 rotor for 16 hr at 16,000 rpm.

Control of infectivity. During purification, aliquots of AAV 5 suspension were taken at individual steps of the procedure (supernatant after removing of cell debris, suspension after dialysis, and resuspended pellet after the SW 27 run and after the SW 41 run) and were assayed for infectivity. The aliquots were heated at 56° for 30 min and then mixed with adenovirus type 12 stock virus as helper. KB cells were infected as described above and particle production was monitored by electron microscopy. Additionally, in some experiments infected cells were incubated for about 20 hr, then harvested and analyzed for the expression of AAV antigens by immunofluorescence (Henle and Henle, 1966). The monoclonal antibody directed against AAV 5 capsid proteins is described elsewhere (Georg-Fries *et al.*, 1984).

Extraction and purification of viral DNA. Pelleted virus was resuspended and the DNA was extracted by treating the sus-

pension with 0.1 *N* NaOH, 0.15% Sarkosyl, and 1 × SSC (0.15 *M* NaCl, 0.015 *M* sodium citrate) for 1 hr at room temperature (Berns *et al.*, 1975). The solution was neutralized with 0.2 *N* HCl and then treated with proteolytic enzymes (trypsin, 10 μg/ml, 1 hr, 27°, 33 U/mg; proteinase K, 10 μg/ml, 1 hr, 37°, 20 U/mg; both enzymes were from Boehringer, Mannheim). Desalting, removal of proteins, and concentration of DNA were done by chromatographing the solution on a small Sephadex G 50/malachite green gel column (Bünemann and Müller, 1978; Koller *et al.*, 1978). Care has to be taken to sufficiently dilute the solution before loading it onto the malachite green gel since higher salt concentrations will hinder the binding of DNA to the affinity material. The buffer used for equilibrating and washing of the column and for diluting the DNA was 10 m*M* Tris, 1 m*M* EDTA, pH 6.5. Elution was done with 2 *M* sodium perchlorate, 10 m*M* Tris, pH 7.5.

Gel electrophoresis. DNA was electrophoresed on agarose (SeaKem) slab gels (Sharp *et al.*, 1973). Separation of fragments of restriction enzyme digest was additionally performed on a 3.5 to 13% polyacrylamide gradient gel (acrylamide:bisacrylamide, 29:1) (Jeppesen, 1980). For preparative purposes, 1% low-melting-temperature agarose (LMT agarose) (SeaPlaque) gels were used. The LMT agarose was sterilized and the gels were poured in the cold room. Gels were run in a buffer of pH 7.8 containing 40 m*M* Tris, 5 m*M* sodium acetate, and 1 m*M* EDTA. PM 2 DNA, *Hin*dIII-cleaved, and φX174 RF DNA, *Hae*III-cleaved, were used as size markers. For staining, the gels were soaked in an ethidium bromide solution (0.5 μg/ml) for 30 min (Sharp *et al.*, 1973).

Preparation of AAV duplex DNA. Annealing was done by heating the DNA eluted from the malachite green gel/Sephadex G 50 column at 65° for 15 min and then slowly cooling the solution to room temperature. The DNA was fractionated by electrophoresis on 1% LMT agarose gels. From these gels DNA was recovered according to the method described by Weislander (1979). If necessary, the DNA was concentrated prior to electrophoresis by sedimenting it in an air-driven ultracentrifuge (Airfuge, Beckman) at 76,000*g* for 75 min. To reduce convection, sedimentation was performed in a solution containing small amounts of CsCl (n_D^{20} = 1.3362). For nick-translation, double-stranded DNA was not extracted from the LMT agarose. Instead the method of Parker and Seed (1980) for sequential digest was applied. The gel slice containing the DNA was cut out and melted by heating it to 65° (buffer, salt, and water added). It then was further diluted by adding the dNTPs at 37° before starting the enzymatic reaction at 17°.

Nick-translation. Nick-translation was achieved by the procedure of Kelly *et al.* (1970) and Rigby *et al.* (1977). Radiolabeled [α-^{32}P]dCTP and [α-^{32}P]dTTP were purchased from Amersham Buchler, Braunschweig.

Transfer of DNA onto nitrocellulose filters. The DNA was transfered to nitrocellulose filters (Schleicher and Schüll, BA 85) as described by Southern (1975). If identical nitrocellulose filters were needed for hybridization with two different probes, bidirectional transfer was performed according to Smith and Summers (1980).

Filter hybridization. Filter hybridization was carried out at 42° according to the technique of Denhardt (1966). The hybridization solution contained 50% formamide, 5 × SSC (0.75 *M* NaCl, 0.075 *M* sodium citrate), 20 m*M* sodium phosphate, pH 6.5, 5 × Denhardt's solution (Denhardt, 1966), and 100 μg/ml t-RNA in addition to the ^{32}P-radiolabeled DNA. After hybridization, the filters were washed at 70° in a solution containing 1 × SSC and 0.1% sodium dodecyl sulfate for 90 min with three subsequent changes of buffer, and then autoradiographed by using a Kodak X Omat AR film with intensifying screen at −70°.

Digestion with endodeoxyribonucleases (restriction enzymes). The enzymes used for digestion of AAV duplex DNA were *Bam*HI, *Eco*RI, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I. With the exception of *Hpa*I and *Kpn*I (New England Biolabs), all others were purchased from Bethesda Research Laboratories. Enzymatic digestions were carried

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out at 37° in final volumes of 10 and 14 μl, respectively. Buffers were used according to the recommendations made by Maniatis *et al.* (1982). For mapping experiments, enzymes were diluted in the appropriate buffers prior to adding them to the DNA solution, so that their activities ranged between 0.8 and 1.5 U. Double digestions were carried out simultaneously if both enzymes were active in the same buffer. Alternatively, the enzyme reactive in a buffer of lower ionic strength was used initially, and then the reaction was continued with the second enzyme after adding the appropriate amount of salt.

RESULTS

Propagation of Virus

Superficial scrapings from a flat condylomatous lesion of the penis were suspended in Eagle's MEM containing 5% FCS and added to a culture of human foreskin fibroblasts (line α1). The cells were coinfected with adenovirus type 12 at an input multiplicity of approximately 10 PFU/cell. The cytopathogenic changes were typical for adenovirus but showed a remarkable delay, with alterations first noted 5 days after infection. Only few adenovirus particles were observed when the supernatant of the pelleted culture fluid was examined by electron microscopy. In addition, however, high concentrations of small particles about 20 nm in diameter were noted. In control cultures infected with adenovirus type 12, no additional virus was discovered. Subcultivation of the virus suspension in human KB cells resulted in a further increase of the small particles within 3 days after infection. No evidence was obtained

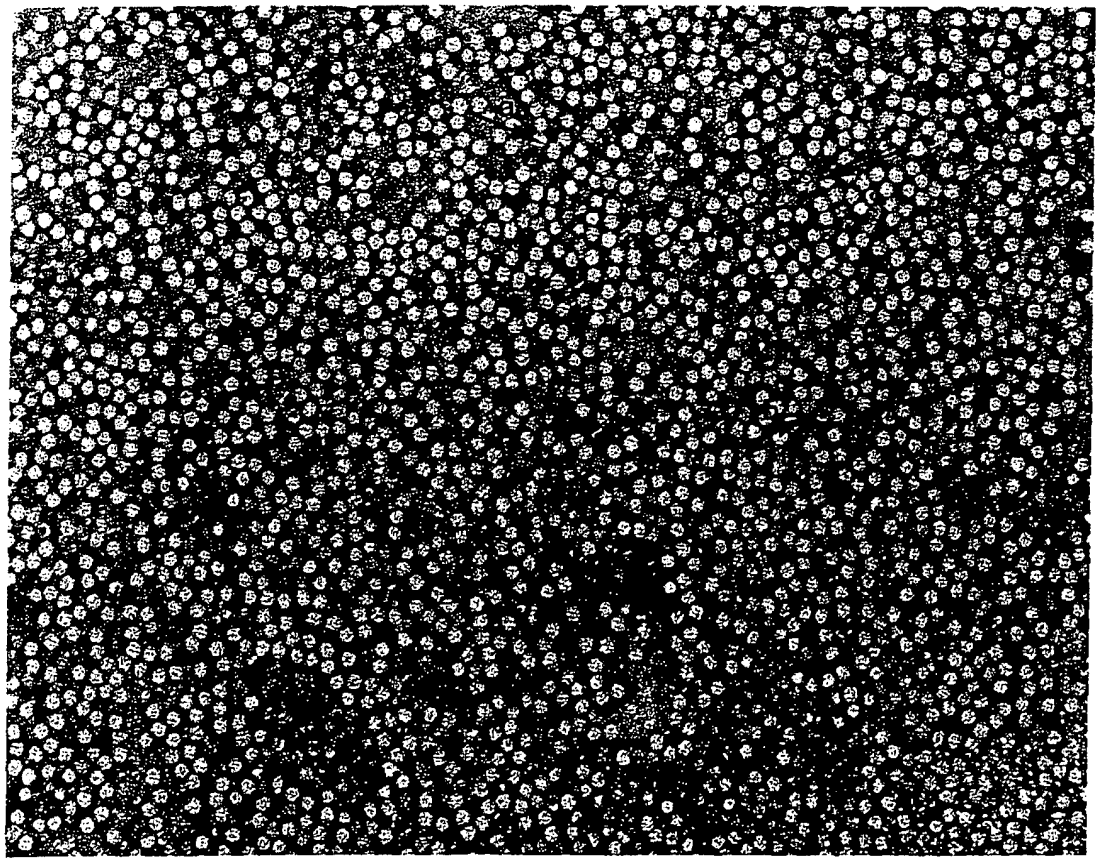

FIG. 1. Electron micrograph of AAV 5 particles. AAV 5 was isolated from infected KB monolayer cells (adenovirus type 12 used as helper) and concentrated by ammonium sulfate precipitation and sedimentation in the ultracentrifuge. The final centrifugation step through low-density CsCl in the SW 41 rotor yields a pellet that contains AAV particles of all density classes ("full" (a) and "empty" (b) particles), and the small capsomers (c) of the destroyed helper virus.

for helper-independent replication of the small virus after prior inactivation of the adenovirus by keeping the virus suspension at 60° for 30 min.

The size of the particles and the apparent dependence on a helper virus for its replication suggested that the virus belongs into the group of defective parvoviruses (adeno-associated viruses, AAV).

Virus Purification

Since adeno-associated viruses are known to consist of a population of various density classes (Hoggan *et al.*, 1966; Hoggan, 1971), conditions for the purification procedure intended to avoid the exclusion of any of these classes. Therefore, the virus suspension was sedimented through a CsCl cushion and finally was pelleted through low-density CsCl in a SW 41 rotor. The pellet of the final centrifugation step thus should contain all kinds of virus particles and, upon DNA extraction, should directly lead to the variant AAV DNAs (Laughlin *et al.*, 1979; de la Maza and Carter, 1980) in addition to DNA of unit length (Fig. 2).

As seen in Fig. 1, virus particles of the adenovirus helper are destroyed during the course of purification, probably due to high salt concentrations and heating. Capsomer units are still present. After application of DNAse, the amounts of contaminating adenovirus DNA are only small (Figs. 2, 3A).

Infectivity of AAV 5 remains intact throughout the purification procedure, as judged by taking the material obtained after different steps for infection tests and then analyzing it by electron microscopy. Similarly, when AAV antigen expression was studied by indirect immunofluorescence the results led to the same conclusion. In either case, no significant decrease of infectious particles was observed after the individual steps of purification (data not shown).

Viral DNA

The majority of DNA eluted from the malachite green gel/Sephadex G 50 column consisted of single strands, as judged from agarose gels (data not shown). Upon annealing, a number of different physical forms were obtained in addition to unit length, linear duplex DNA. These forms were known to contain unit length, duplex circules and linear duplex dimers besides a number of other less well-defined structures (Gerry *et al.*, 1973; Koczot *et al.*, 1973). Heating at 65° for 15 min followed by slow cooling to room temperature converted the single-stranded DNA into the duplex form but, additionally, lead to formation of structures that did not enter a 1% agarose gel (Fig. 2). This phenomenon was also seen when the DNA was concentrated by precipitation or sedimentation in an air-driven ultracentrifuge, and was probably due to oligomeric network-like structures of AAV DNA originating in properties contributed by the terminal structures (Gerry *et al.*, 1973; Koczot *et al.*, 1973).

During the purification of virus no selection was made with regard to particle density. Thus, after DNA extraction molecules of less than genome length were present originating from defective interfering particles (de la Maza and Carter, 1980). As can be seen from Fig. 2, this vari-

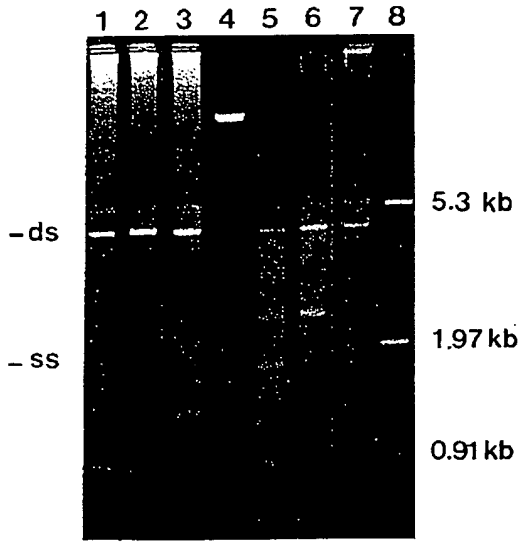

FIG. 2. Fractionation of AAV DNAs in agarose gels. The DNAs of different types of AAV were extracted from the virus particles as described. After heating to either 37° (lanes 5, 6, 7) or 65° (lanes 1, 2, 3) with subsequent slow cooling to room temperature, they were electrophoretically separated in a 1% agarose gel. Lanes 1 and 5, AAV 5 DNA; lanes 2 and 6, AAV 3 DNA; lanes 3 and 7, AAV 1 DNA; lane 4, adenovirus type 12 DNA; lane 8, PM 2 DNA, *Hin*dIII-cleaved as size marker. ds, unit-length duplex monomers; ss, single-strand DNA; kb, 10^3 bp.

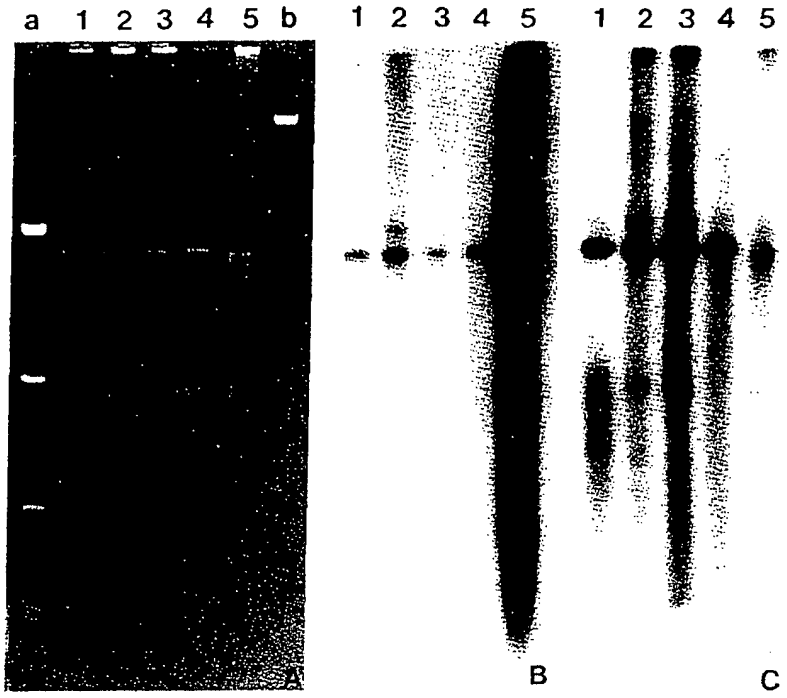

FIG. 3. Comparison of the DNAs of the different AAV serotypes. (A) Fractionation of noncleaved AAV DNAs in a 1% agarose gel stained with ethidium bromide (0.5 μg/ml). (B) Hybridization of one nitrocellulose filter obtained from the same gel after bidirectional Southern transfer with ^{32}P-labeled AAV 5 duplex DNA. (C) Hybridization of the identical filter with ^{32}P-labeled AAV 3 duplex DNA. Prior to radiolabeling, the DNA was fractionated on LMT agarose gels and the duplex DNA then was nick-translated directly from the gel as described. Lane a, PM 2 DNA, *Hin*dIII-cleaved as size marker; lane b, adenovirus type 12 DNA; lane 1, AAV 1 DNA; lane 2, AAV 2 DNA; lane 3, AAV 3 DNA; lane 4, AAV 4 DNA; lane 5, AAV 5 DNA.

ant DNA exhibited a pattern of distinct bands in an agarose gel. The pattern remained constant for repeated virus propagation (data not shown), suggesting that distinct types of variant DNA were either preferentially made or/and encapsidated in the virus particles.

Comparison of the DNAs of the Different AAV Serotypes

Comparison of the AAV 5 DNA with the DNAs of the four serotypes AAV 1–AAV 4 in agarose gels revealed that the AAV 5 DNA was of a slightly smaller size (4.5 to 4.6 kb as calculated from the migration distance, Fig. 3A). As expected, the banding pattern representing the variant DNAs of defective interfering particles (de la Maza and Carter, 1980) was different for each serotype (Fig. 2). Preparations of AAV 5 DNA contained far more DNA of a size smaller than unit length than seen, e.g., in AAV 3 DNA preparations (Fig. 2). Upon reannealing, this led to a heterogenous population of molecules in addition to the predominant species that exhibited the distinct banding pattern. In hybridization experiments with the homologous ^{32}P-labeled DNA, this resulted in labeling of the complete lane on the filter. As expected from the smaller quantity of variant DNA, this phenomenon was less pronounced with AAV 3 (Figs. 3B, lane 5, and C, lane 3).

In blot-hybridization experiments with ^{32}P-radiolabeled AAV 5 duplex DNA, only a weak cross-hybridization with the other four serotypes was seen under stringent conditions. This cross-reactivity was essentially confined to the bands representing complete genomes (Fig. 3B). Additionally, a very weak signal appeared at the position where the variant DNA of the largest size migrated when long times of exposure were used. A similar picture emerged with regard to AAV 5 DNA when hybridization of an identical filter was

performed with ^{32}P-labeled AAV 3 duplex DNA (Fig. 3C). The cross-hybridization occurred at the positions representing the complete AAV 5 genome and the variant AAV 5 DNA of the largest size. Obviously, the serotypes AAV 1-AAV 4 shares more homologies with each other than they do with AAV 5, since all of them rather strongly cross-hybridized with AAV 3 duplex DNA. In addition, the reaction was extended to far more of the variant DNAs than in the case of AAV 5 (Fig. 3C). These findings were supported by the results shown in Fig. 5B. While ^{32}P-labeled AAV 3 duplex DNA showed cross-hybridization with all fragments obtained from restriction-enzyme digested AAV 2 DNA, it did not react with the terminal fragments of AAV 5 DNA (*Xho*I B-fragment, *Bam*HI C-fragment, cf. Fig. 7).

Digesting the isolated double-stranded AAV DNAs with restriction enzymes yielded different restriction patterns for all five serotypes, as seen in Figs. 4 and 5. The cleavage sites observed with the endonucleases *Bam*HI and *Xho*I for the new isolate clearly differed from those seen for AAV 1, AAV 2, AAV 3, and AAV 4 (Figs. 4, 5). In addition, no identical cleavage pattern was obtained when the DNAs of AAV 1, AAV 3, AAV 4, and AAV 5 were digested by the enzyme *Hinc*II (Fig. 4).

Restriction Enzyme Patterns and Size of Fragments for AAV 5 DNA

Isolated AAV 5 duplex DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hinc*II, *Hind*III, *Hpa*I, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I. Within the resolution of a 2% agarose gel, no cleavage site has been found with the enzymes *Hind*III, *Hpa*I, *Kpn*I, *Pst*I, and *Xba*I. For the other restriction enzymes used, the number of fragments and their sizes, as estimated from 2% agarose gels (data not shown) and 3.5 to 13% polyacrylamide gels (Fig. 6), are listed in Table 1. With the technique used (Southern transfer and filter hybridization) the smallest fragment seen from agarose gels in X-ray films was about 220 bp in size (*Sma*I C-fragment), and in some cases the *Xho*I C-fragment with a size of about 140 bp could

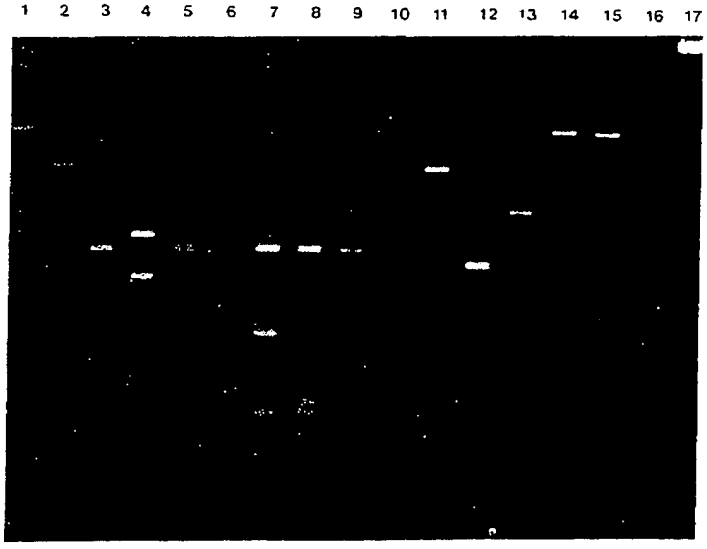

FIG. 4. Digestion of AAV DNAs with restriction enzymes. The duplex DNA of AAV 1, AAV 3, AAV 4, and AAV 5 was isolated from agarose gels and cleaved with the restriction enzymes *Bam*HI, *Hinc*II, and *Xho*I at 37° in a final volume of 14 μl. Lanes: 1. AAV 5, uncleaved; 2. AAV 5, *Xho*I; 3. AAV 4, *Xho*I; 4. AAV 3, *Xho*I; 5. AAV 1, *Xho*I; 6. AAV 5, *Hinc*II; 7. AAV 4, *Hinc*II; 8. AAV 3, *Hinc*II; 9. AAV 1, *Hinc*II; 10. AAV 5, *Bam*HI; 11. AAV 4, *Bam*HI; 12. AAV 3, *Bam*HI; 13. AAV 1, *Bam*HI; 14. AAV 4, uncleaved; 15. AAV 3, uncleaved; 16. AAV 1, uncleaved; 17. adenovirus type 12 DNA, uncleaved.

FIG. 5. Digestion of AAV 2 DNA and AAV 5 DNA with restriction enzymes. The duplex DNA of AAV 2 and AAV 5 was isolated and cleaved with the restriction enzymes *Bam*HI and *Xho*I as described in Fig. 4. The fragments were bidirectionally transfered to nitrocellulose filters and the filters were hybridized with ^{32}P-labeled AAV 5 DNA (A) and AAV 3 DNA (B). Since the cross-hybridization between AAV 3 DNA and AAV 5 DNA is rather weak (cf. Fig. 3C), the AAV 5 DNA was used in higher concentrations. Thus the cross-hybridization between AAV 2 DNA and AAV 5 DNA is not seen at the time of exposure shown in (A). Lanes: 1. AAV 2, uncleaved; 2. AAV 5, uncleaved; 3. AAV 2, *Bam*HI; 4. AAV 5, *Bam*HI; 5. AAV 2, *Xho*I; 6. AAV 5, *Xho*I.

be seen after prolonged times of exposure. The lower limit for fragment size still detectable unambiguously from blotted polyacrylamide gels was about 100 bp (Fig. 6, lane 15) and with this, the smallest fragment found with the single enzyme digest was indeed the *Xho*I C-fragment.

Mapping of Restriction Enzyme Cleavage Sites on AAV 5 Duplex DNA

Mapping of restriction enzyme cleavage sites was accomplished for the enzymes *Bam*HI, *Eco*RI, *Hinc*II, *Sal*I, *Sma*I, and *Xho*I. Double and partial digests were performed and the resulting fragments were separated on agarose gels (data not shown) and polyacrylamide gradient gels (Fig. 6), from which they were transferred to nitrocellulose filters and visualized by hybridization with ^{32}P-labeled AAV 5 DNA.

The map derived from these data is shown in Fig. 7. As the minus strand of AAV 5 DNA has not yet been determined, no orientation of the map according to the

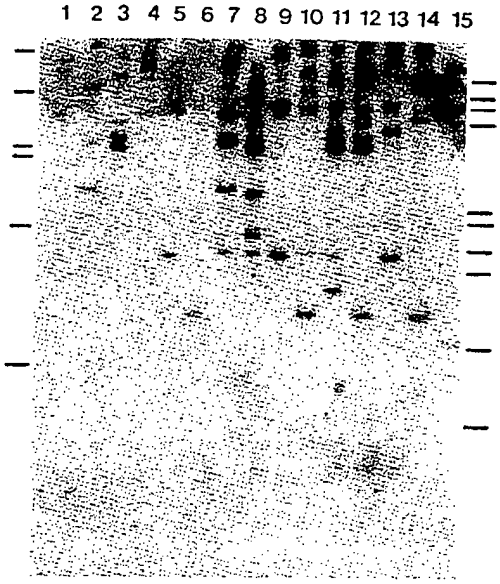

FIG. 6. Cleavage of AAV 5 double-stranded DNA with different restriction enzymes. AAV 5 duplex DNA was isolated and cleaved with different restriction enzymes at 37° for 1 hr in a final volume of 10 μl. Enzymatic activities in the test were between 0.8 and 1.5 U. Buffers were as mentioned in the text. The fragments were separated in a 3.5 to 13% polyacrylamide gel with a 3% stacking gel, and then transfered to nitrocellulose filters as described and visualized by hybridization with ^{32}P-radiolabeled AAV 5 DNA. Size markers were PM 2 DNA, *Hind*III-cleaved (left track) and φX174 RF DNA, *Hae*III-cleaved (right track) (cf. Table 1), with a resolution of 1.97 to 0.1 kb (PM 2 marker) and 1.35 to 0.072 kb (φX174 marker). The C-fragment of the *Sma*I digest still seen in lane 11 (double-digest *Sma*I and *Hinc*II) is due to incomplete digest since it usually was not seen in polyacrylamide gels of this double digest. Lanes: 1. *Bam*HI; 2. *Eco*RI; 3. *Hinc*II; 4. *Sal*I; 5. *Sma*I; 6. *Xho*I; 7. *Bam*HI and *Eco*RI; 8. *Bam*HI and *Hinc*II; 9. *Bam*HI and *Sma*I; 10. *Bam*HI and *Xho*I; 11. *Hinc*II and *Sma*I; 12. *Hinc*II and *Xho*I; 13. *Sal*I and *Sma*I; 14. *Sal*I and *Xho*I; 15. *Sma*I and *Xho*I.

TABLE 1

SIZE OF AAV 5 DNA RESTRICTION FRAGMENTS AS ESTIMATED FROM 2% AGAROSE GELS AND 3.5 TO 13% POLYACRYLAMIDE GELS

	Fragment size (base pairs × 10^3)						
Enzyme	A	B	C	D	E	F	Total
*Bam*HI	1.85	1.40	1.03	0.23	—	—	4.51
*Eco*RI	2.65	1.10	0.47	0.34	—	—	4.56
*Hinc*II	1.32	0.95	0.65	0.56	0.50	0.50	4.48
*Sal*I	2.95	1.60	—	—	—	—	4.55
*Sma*I	3.55	0.78	0.22	—	—	—	4.55
*Xho*I	3.65	0.76	0.14	—	—	—	4.55

Note. PM 2 DNA, *Hin*dIII-cleaved and the DNA of ϕX174 RF, *Hae*III-cleaved, were used as size markers. Fragment size of marker DNA (base pairs × 10^3): PM 2 DNA, *Hin*dIII-cleaved; 5.3, 1.97, 0.91, 0.44, 0.42, 0.26, and 0.1. ϕX174 RF DNA, *Hae*-III-cleaved; 1.35, 1.08, 0.87, 0.6, 0.31, 0.271/0.281, 0.234, 0.194, 0.118, and 0.072.

convention by Ward and Tattersall (1978) was made. Since the smallest fragment that could be identified with certainty by the technique used was about 100 bp in size, the small fragments resulting from the double digests *Hinc*II/*Sma*I (about 50 bp) and *Hinc*II/*Xho*I (about 50 bp), and one fragment expected from the *Sma*I/*Xho*I digest (about 20 bp) were not seen.

DISCUSSION

The experiments presented in this report describe a new type of a helper-dependent parvovirus. With regard to its helper dependence and its DNA structure, this virus clearly belongs into the group of adeno-associated viruses and is tentatively labeled as AAV 5.

Adeno-associated viruses are known to give rise to several visible bands when centrifuged to equilibrium in CsCl buoyant density gradients (Hoggan *et al.*, 1966; Hoggan, 1971). In addition to these bands, the spectrum of virus particles is also extended to the interbanding regions (de la Maza and Carter, 1980). We therefore chose a purification procedure that avoids exclusion of any density group. Thus, upon DNA

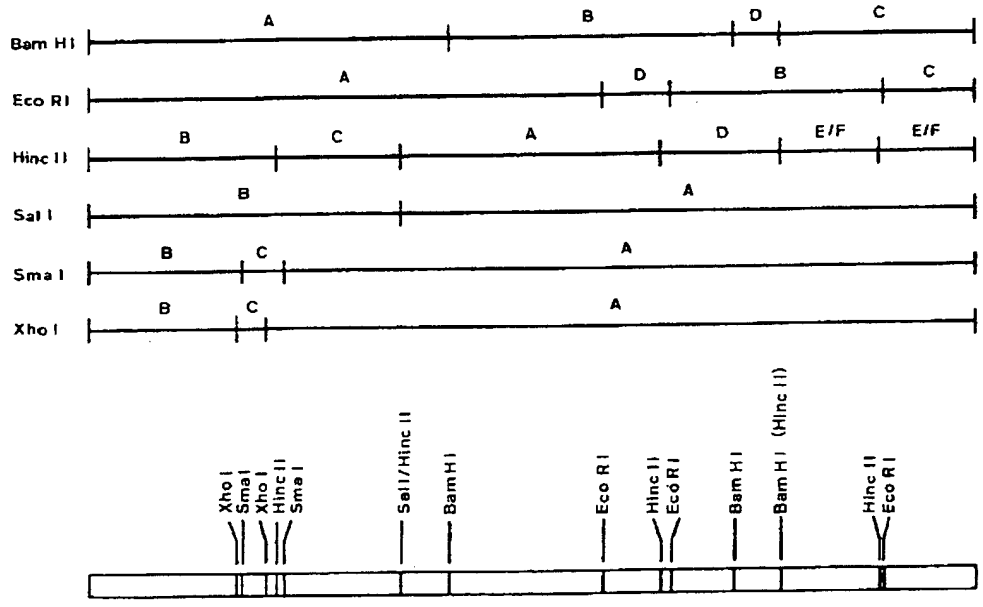

FIG. 7. Map of restriction endonuclease cleavage sites in the DNA of AAV 5. The cleavage sites of the restriction endonucleases *Bam*HI, *Eco*RI, *Hinc*II, *Sal*I, *Sma*I, and *Xho*I were determined. Fragments are designated by alphabetic letters. Their respective size is listed in Table 1. The fragments E and F of the *Hinc*II cleavage comigrate in the agarose gel and are also not resolved in the polyacrylamide gel. Thus, they were not distinguished. The cleavage sites for the enzyme *Bam*HI (D/C) and for the enzyme *Hinc*II (D/E(F)) are not resolved since they are very close to each other.

extraction the whole population of AAV DNA molecules was obtained (Fig. 2). Since the variant DNA (de la Maza and Carter, 1980) leads to a distinct banding pattern in agarose gels (Fig. 2), it is possible to directly extract specific forms of AAV DNA from the gels. The banding pattern exhibited by the variant DNAs is characteristic for each individual serotype and remains constant upon repeated virus propagation. This might originate either from the mechanism of DNA synthesis or from a site-specific cleaving of the DNA, or else from preferential encapsidation of defined species of molecules.

Comparison of the DNA of the newly isolated defective parvovirus (AAV 5) with the DNAs of the other four AAV serotypes by blot-hybridization experiments shows only a weak cross-reactivity (Fig. 3B). This is confined to the DNA bands representing complete genomes and, to a lesser extent, to the variant DNA of the largest size. On the other hand, rather strong cross-hybridization is seen between the other four AAV serotypes, and this reaction extends to smaller variant DNAs as well (Fig. 3C). These findings not only show that the isolated parvovirus is indeed different from AAV 1–AAV 4, but also demonstrate that AAV 5 is more distantly related to the four adeno-associated viruses than these are among each other. Since the variant DNAs show increasing deletions for internal regions as their size decreases but retain the genome termini (de la Maza and Carter, 1980), the homologies in the DNA of AAV 5 and AAV 1–AAV 4 are obviously located within the internal region. This assumption is supported by the finding that radiolabeled AAV 3 DNA shows no cross-hybridization with the terminal B-fragment of the *Xho*I cleavage and the terminal C-fragment of the cleavage with the enzyme *Bam*HI (Figs. 5, 7).

The concentration of unit length, linear duplex DNA in individual preparations usually is rather small (Fig. 2), and therefore a sensitive method was needed to determine the fragments obtained by digesting the double-stranded DNA with restriction enzymes. Since it was possible to transfer the DNA from polyacrylamide gels to nitrocellulose filters (with experimental problems occurring only at low polyacrylamide concentrations) and to visualize it by filter hybridization, fragments down to 100 bp could be detected (Fig. 6). With this, no terminal fragments of the AAV 5 DNA smaller than the *Xho*I B-fragment (760 bp) and the *Eco*RI C-fragment (470 bp) were found for the enzymes tested, and mapping of the terminal region of the AAV 5 DNA remains to be done.

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